ORIGINAL ARTICLE

Clinical application of a systems model of apoptosis execution for the prediction of colorectal cancer therapy responses and personalisation of therapy

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ABSTRACT

Objective Key to the clinical management of colorectal cancer is identifying tools which aid in assessing patient prognosis and determining more effective and personalised treatment strategies. We evaluated whether an experimental systems biology strategy which analyses the susceptibility of cancer cells to undergo caspase activation can be exploited to predict patient responses to 5-fluorouracil-based chemotherapy and to case-specifically identify potential alternative targeted treatments to reactivate apoptosis.

Design We quantified five essential apoptosis-regulating proteins (Pro-Caspases 3 and 9, APAF-1, SMAC and XIAP) in samples of Stage II (n=13) and III (n=17) tumour and normal colonic (n=8) tissue using absolute quantitative immunoblotting and employed systems simulations of apoptosis signalling to predict the susceptibility of tumour cells to execute apoptosis. Additional systems analyses assessed the efficacy of novel apoptosis-inducing therapeutics such as XIAP antagonists, proteasome inhibitors and Pro-Caspase-3-activating compounds in restoring apoptosis execution in apoptosis-incompetent tumours.

Results Comparisons of caspase activity profiles demonstrated that the likelihood of colorectal tumours to undergo apoptosis decreases with advancing disease stage. Systems-level analysis correctly predicted positive or negative outcome in 85% (p=0.004) of colorectal cancer patients receiving 5-fluorouracil based chemotherapy and significantly outperformed common uni- and multi-variate statistical approaches. Modelling of individual patient responses to novel apoptosis-inducing therapeutics revealed markedly different inter-individual responses.

Conclusions Our study represents the first proof-of-concept example demonstrating the significant clinical potential of systems biology-based approaches for predicting patient outcome and responsiveness to novel targeted treatment paradigms.

Significance of this study

What is already known about this subject?

- There are no effective response markers to non-targeted chemotherapies for colorectal cancer.
- Effective treatment for colorectal cancer using traditional chemotherapies, such as 5-fluorouracil and oxaliplatin, is hindered due to chemoresistance, due in part to defective apoptosis.
- Due to its important role in cancer progression and chemotherapy response, the intrinsic apoptosis pathway and its associated proteins represent both potential response markers and points of therapeutic intervention.

What are the new findings?

- This proof of concept study indicates that computationally modelling of non-linear apoptosis signalling can predict response to 5-fluorouracil based chemotherapy with high accuracy, outperforming traditional statistical methods based on tumoural concentrations of proteins involved in apoptosis execution.
- Extending the model to model individual patient responses to novel targeted therapeutics (XIAP antagonists, proteasome inhibitors and Pro-Caspase 3-activators) demonstrated varied inter-individual responses to these agents.

How might it impact on clinical practice in the foreseeable future?

- This study indicates the potential of systems biology-based approaches in predicting patient outcome and responsiveness to novel targeted chemotherapies. This study highlights how such approaches may enable oncologists to make more informed treatment decisions, and to provide reasoning for the inclusion of alternative, adjuvant treatment paradigms.
- While this study focuses on the clinical potential of this systems modelling approach in colorectal cancer, it may have similar clinical potential when applied to other malignancies.

INTRODUCTION

Throughout the world, cancer is a leading cause of death with incidence and mortality projected to rise. Colorectal cancer is the fifth most common form of cancer and third leading cause of cancer related mortality. Current treatment options for non-metastatic Stage II and III colorectal cancer consist of surgical resection with or without 5-fluorouracil (5-FU) based chemotherapy. While survival rates have improved through the use of

5-FU/oxaliplatin combination chemotherapy, not all patients will benefit due to either inherent or acquired chemoresistance.[1]

The major molecular changes occurring during colorectal tumourigenesis are well understood, including both epigenetic and mutational events leading to genetic instability and the malignant phenotype.[2] A common result of these changes is that they confer a survival advantage, leading to impaired apoptosis and thereby contributing to uncontrollable cell growth and resistance to traditional chemotherapies such as the DNA damaging agents 5-FU and oxaliplatin. The intrinsic pathway of apoptosis (figure 1) is characterised by mitochondrial outer membrane permeabilization (MOMP) and is activated in response to cellular stress resulting from DNA damaging chemotherapeutics and radiation therapy.[4 5] MOMP leads to cytochrome-c and SMAC release from mitochondria followed by apoptosome formation and caspase activation. Specifically, cytochrome-c complexes with APAF-1, recruiting and activating the initiator Caspase 9 which goes on to activate the effector caspases, in particular Caspase 3 leading to the biochemical and morphological changes characteristic of apoptosis.[6] Caspase activity is inhibited by XIAP,[7] whose action is antagonised through its interactions with the pro-apoptotic SMAC.[8] Caspase activation following cytochrome-c and SMAC release occurs within minutes; however this rapid response is significantly slowed when XIAP levels are high[3] which may be particularly important in cancers as XIAP is overexpressed in a number of tumour types.[9–11]

Due to its importance in drug response and resistance, proteins associated with apoptosis are frequently examined as prognostic indicators in colorectal cancer. APAF-1,[12 13] SMAC,[12 14] XIAP,[11] and the Caspases 3[15] and 9[16] have been shown to be differentially expressed between colorectal tumours and matched normal tissue, with increased expression of APAF-1[13] and SMAC[14] and decreased XIAP[11] expression associating with longer patient survival. However, it is important to note that the decision between apoptosis and survival is not determined solely by individual proteins but rather by the relative abundance and interactions of multiple key apoptotic proteins. Therefore examination of the system as a whole rather than single markers may be more informative for patient prognosis and in identifying targets for therapeutic intervention. Using mathematical modelling we previously demonstrated that the decision to undergo apoptotic cell death may be determined with high precision using sets of ordinary differential equations (ODEs) which reflect the non-linear processes of apoptosis execution.[3] This computational systems model, APOPTO-CELL, is based on the concentrations of five key proteins involved in mitochondria mediated apoptosis (specifically the Pro-Caspases 3 and 9, SMAC, APAF-1, and XIAP).[3 17] Originally developed in HeLa cells and validated experimentally in cellulo,[3] the potential for this model in the clinical setting has not yet been determined. In this study we examined its ability to predict differences in caspase substrate cleavage profiles between colorectal tumours and normal tissue. Furthermore, we explored the potential for this model to predict chemotherapy responses of dormant mircometastatic sites to 5-FU-based chemotherapy based on an analysis of the primary tumour site, and to identify personalised alternative treatment options for colorectal cancer patients. Taken together, this study is one of the first studies to validate the clinical potential of ODE systems biology models, indicating an important milestone in translational medical systems biology.

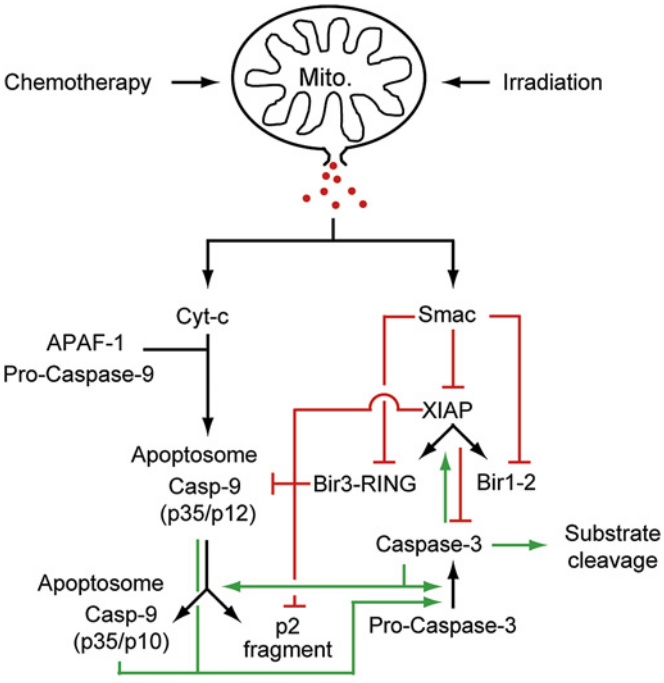

Figure 1 Apoptosis pathway following mitochondrial permeabilization in response to DNA damage. Following permeabilization of the mitochondrial outer membrane cytochrome-c and SMAC (red dots) are released into the cytosol, initiating the apoptosis execution network modelled by the ODE-based APOPTO-CELL model.[3]

MATERIALS AND METHODS

A description of quantitative western blotting and gene expression by real time quantitative RT-PCR is provided in Supplementary text.

Patient cohort

Colorectal cancer patient tissue was collected and stored from the Departments of Surgery, Gastroenterology and Pathology, Beaumont Hospital, Dublin, Ireland. Pathologic stage was determined using Dukes' and TNM staging. Surgically resected colorectal tumours were obtained from 30 Stage II (n=13) and Stage III (n=17) patients. Matched adjacent normal colorectal tissue was available for 26 of these patients. Patient characteristics are summarised in table 1. Twenty of these patients received 5-FU- based chemotherapy, specifically 5-FU/leucovorin (n=17), 5-FU/oxaliplatin/leucovorin (n=2) and 5-FU/irinotecan/leucovorin (n=1). Clinical follow-up was obtained through a review of medical records by a dedicated clinical research nurse. For classification purposes, patients without disease recurrence and/or cancer mortality within 4 years were classified as a 'positive outcome'; patients who recurred/died from colorectal cancer were classified as 'negative outcome'. Patients with hereditary forms of colorectal cancer were excluded. Ethical approval has been obtained for this study by the Beaumont Hospital Ethics (Medical Research) Committee and informed consent was obtained from all patients. Tissue was stored for use as snap frozen (−80°C) or in RNAlater (Ambion, Abington, UK) (−20°C).

Statistical analysis

Wilcoxon signed—rank tests were performed to identify significant differences between tumour and normal protein expression relative to β-actin. Correlations between mRNA and protein expression was assessed using Pearson correlation. Associations

Table 1 Patient clinical characteristics

	Stage II	Stage III	Stages II and III
Median age (years)	73.5	61.3	68.25
Gender			
Male	9	11	20
Female	4	6	10
Chemotherapy received			
Yes	3	17	20
No	10	0	10
Tumour location			
Caecal	1	6	7
Ascending colon	3	2	5
Transverse colon	1	0	1
Descending colon	1	0	1
Sigmoid	3	5	8
Rectosigmoid	2	2	4
Rectal	2	2	4
Microsatellite instability status*			
Yes	2	0	2
No	5	13	18
Median survival time (months)			
Disease free survival	20	16	20
Overall survival	25	20	22.5

*MSI status assessable in 20/30 patients.

between model output parameters and patient outcome were determined using Mann–Whitney U tests.

In order to assess the ability of these proteins to predict patient outcome, univariate discriminant analysis with cross-validation was carried out, with each protein analysed separately for its ability to predict outcome. To determine whether the concentrations of APAF-1, XIAP, SMAC and Pro-Caspases 3 and 9 taken in combination associated with clinical outcome, multivariate statistical methods were used. Stepwise forward logistic regression was used to determine whether co-variates such as microsatellite instability (MSI), type of chemotherapy received and tumour differentiation were significant co-variates. Discriminant analysis with cross-validation and principal component analysis (PCA) were utilised, including all proteins as variables in each method. The specificity of the computational model to correctly identify patients with negative outcome following 5-FU based chemotherapy was determined as described by Altman and Bland.[18] Fisher's exact tests were used to determine p values for the results obtained by computational modelling and discriminant analysis. PCA was carried out in MATLAB (Mathworks, Natick, Massachusetts, USA). All other statistical analyses were carried out using SPSS for Windows 15.0 (SPSS Inc.). A p value of <0.05 was considered to be statistically significant.

Mathematical modelling of caspase activation

The APOPTO-CELL model is based on a set of ODEs representing a reaction network of 53 reactions, 19 reaction partners and 75 reaction parameters and is implemented in MATLAB. Parameters were obtained from the literature or by independent analysis.[3 17] The model was originally developed in HeLa cells and validated experimentally in cellulo using genetic manipulations as well as by adapting the model to cancer cell lines deficient in apoptosis signalling proteins.[3 19] The model's output function (substrate cleavage by Caspase 3) is calculated by direct integration of the effector caspase activation profile. Substrate cleavage profiles were generated by implementing concentrations of APAF1, Pro-Caspase 3, Pro-Caspase 9, XIAP and SMAC proteins into an automated version of the model and modelled over 300 min. At 300 min, varied caspase activation profiles were predicted, ranging from <1% to 100%. We have previously demonstrated that cancer cells showing <25% caspase-mediated substrate cleavage showed no discernable signs of apoptosis.[3] Therefore <25% predicted substrate cleavage at 300 min was classified as unlikely to undergo apoptosis.

Implementation of alternative therapy regimes into APOPTO-CELL

The systems model was extended to predict the effects of targeted therapies on the likelihood of tumour samples to undergo caspase activation. Therapeutics included XIAP antagonists,[20] proteasome inhibitors,[21] and Pro-Caspase 3-activating compounds.[22] XIAP antagonists were considered at initial cytosolic concentrations of 100, 250, or 500 nM and mimicking the behaviour of SMAC. Proteasome inhibition was modelled to reduce the degradation rate of proteins by 10%, 50% or 100%. Pro-Caspase 3-activating compounds were modelled at initial concentrations of 50, 100 and 500 nM and assumed to activate Pro-Caspase 3 by mass action kinetics with a k^{cat} of 0.068 μM/min.[22] All simulations were modelled for 300 min following MOMP.

RESULTS

Alterations in apoptosis protein levels in colorectal cancer tumours

Alterations in the apoptotic machinery of cancer cells may contribute to the decreased capacity of tumours to undergo programmed cell death. We first examined whether APAF-1, XIAP, Pro-Caspases 3 and 9, and SMAC proteins were differentially expressed between colorectal tumours and matched normal colorectal tissue from Stages II and III patients using western blotting and quantitative analysis of high dynamic range luminescence images (figure 2). Western blotting demonstrated that there was no differential expression of β-actin protein expression between tumour and matched normal tissue. Colorectal tumour and normal tissue was collected and stored between the years 2002 and 2006 according to procedures established within the Departments of Surgery and Pathology. Inclusion criteria were based on the following parameters: pathologic stage of either Stages II or III, sufficient quality and quantity of stored clinical material, and the availability of complete clinical follow-up data for at least 4 years. Surgically resected colorectal tumours were obtained from 13 Stage II and 17 Stage III patients who met all inclusion criteria, with matched normal tissue available for 26 patients. Twenty of these patients underwent 5-FU based adjuvant chemotherapy following surgery. Patient characteristics are summarised in table 1.

Statistical analysis of protein expression relative to β-actin indicated significantly lower expression of APAF-1 in tumour compared to normal tissue (Wilcoxon rank sum; p=0.026), with 16 of 26 patients showing lower expression in tumour tissue. No significant differences in expression of XIAP (p=0.086), SMAC (p=0.929), Pro-Caspase 3 (p=0.585) and Pro-Caspase 9 (p=0.096) were detected.

We also addressed whether gene expression in tumours correlated with protein expression in patients where the nucleic acid preservative RNAlater stored tissue was available. Importantly we found no significant correlation (Pearson correlation coefficients ranging between −0.536 and 0.029; p values ranging from 0.171 and 0.945) between gene expression and protein

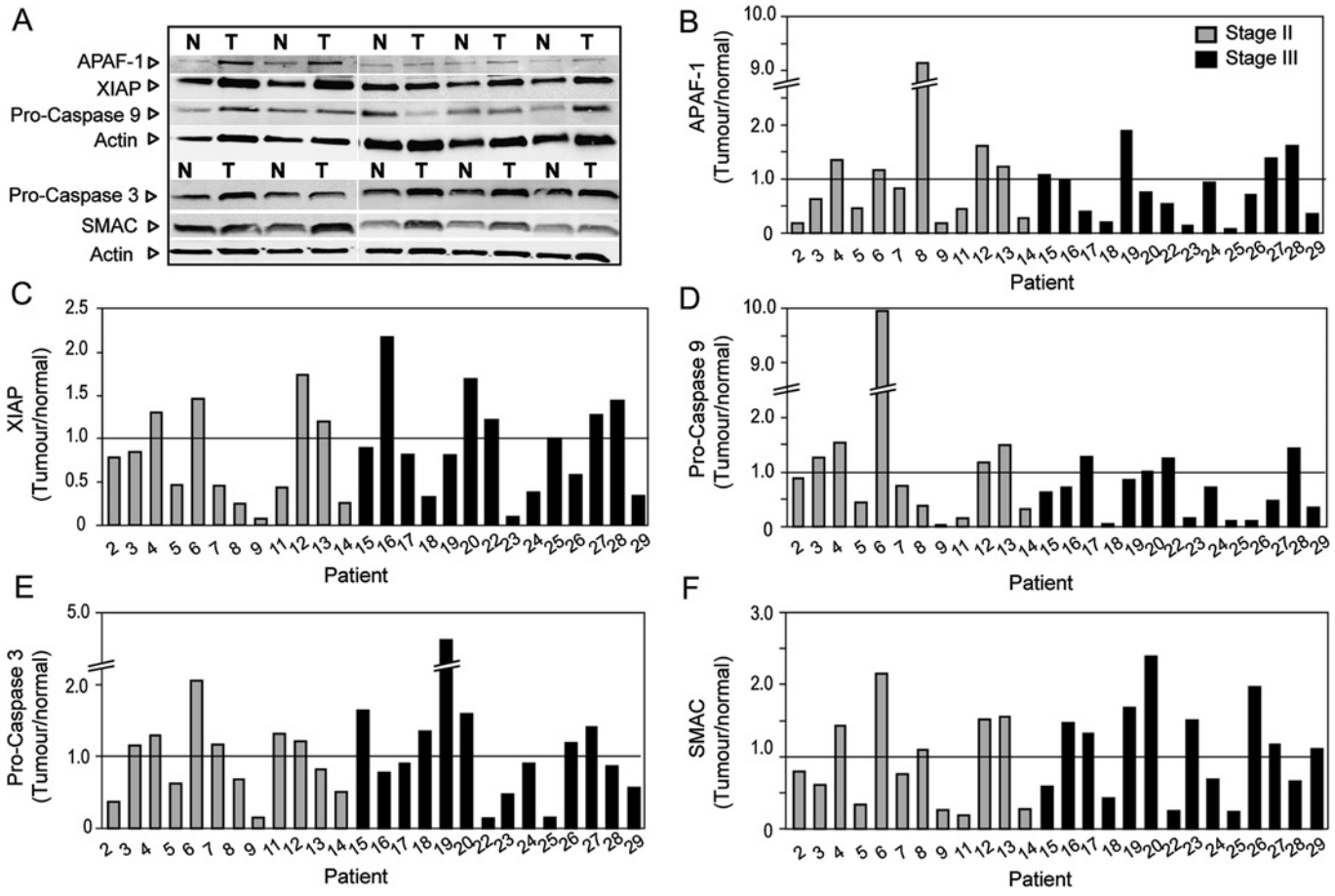

Figure 2 Expression of apoptosis associated proteins in colorectal tumours and adjacent matched normal colorectal tissue. (A) Representative western blots of tumour (T) and matched normal (N) tissue for APAF-1, XIAP, Pro-Caspase 9, Pro-Caspase-3, and SMAC. Graphs represent tumour to normal ratios of APAF-1 (B), XIAP (C), Pro-Caspase 9 (D), Pro-Caspase 3 (E) and SMAC (F) protein expression in matched colorectal tumour and normal tissue from 26 patients. Grey bars represent Stage II patients (n=12), filled bars represent Stage III patients (n=14). Line represents a Tumour/Normal ratio of 1. Patient numbers correspond between all figures and Supplementary figures.

concentrations (Supplementary figure 1), indicating that mRNA levels cannot be used as a surrogate for protein expression levels.

Systems analysis indicates that apoptosis execution is impaired with advancing disease stage

Previously we developed a computational ODE systems model of apoptosis execution that predicts the ability of cancer cells to undergo Caspase 3 activation following the release of cytochrome-c and SMAC.[3] To apply this model, we determined absolute protein concentrations of APAF-1, Pro-Caspase 3, Pro-Caspase 9, XIAP and SMAC in colorectal tumours of Stage II (n=13) and Stage III (n=17) patients using quantitative western blotting (Supplementary figure 2). Protein concentrations were also determined in a subset of normal colorectal tissue from Stage III colorectal cancer patients (n=8) (Supplementary figure 3). Cytochrome-c levels were determined but not included as very small quantities of cytochrome-c are sufficient to activate the apoptosome by transiently binding to APAF-1.[23 24] The effects of tumour heterogeneity were addressed by comparing concentrations of these proteins in different sections of individual tumours, detecting little intra-individual variations compared with inter-individual variations (Supplementary figure 4).

Protein concentrations of APAF-1, XIAP, SMAC and Pro-Caspases 3 and 9 were then implemented into the APOPTO-CELL model and substrate cleavage by effector caspases was plotted to assess for differences in the ability of tumour and normal tissue to undergo apoptosis. In normal colorectal tissue, the model predicted rapid caspase activation following stress-induced MOMP (figure 3A), consistent with previous studies showing rapid activation of caspases following MOMP.[3 25] In tumour tissue, 2 of 13 (15%) Stage II patients and 4 of 17 (24%) of Stage III patients were predicted as unable to undergo caspase activation (figure 3B,C; green traces), indicating a trend towards impaired apoptosis execution with advanced disease stage. Indeed, caspase activity was significantly higher in normal compared with tumour tissue with mean substrate cleavage rates (maximum slope) of 3.37±2.75% and 0.41±0.53% substrate cleavage/min, respectively (Mann–Whitney U test; $p<0.001$). Interestingly, there was no difference in the substrate cleavage rates between Stages II and III patients (Stage II 0.38±0.46%; Stage III 0.43±0.60%; Stage II vs Stage III: $p>0.1$; Stage II vs Normal: $p=0.001$; Stage III vs Normal: $p<0.0001$).

Model prediction of caspase activation correlates with colorectal cancer patient response to chemotherapy

Resistance to chemotherapy may be in part due to resistance to apoptosis. On the assumption that tumours released cytochrome-c and SMAC following chemotherapy, we assessed whether model predictions of caspase activation correlated with clinical outcome in patients who received adjuvant chemotherapy (figure 4). Specifically, we used the model output from

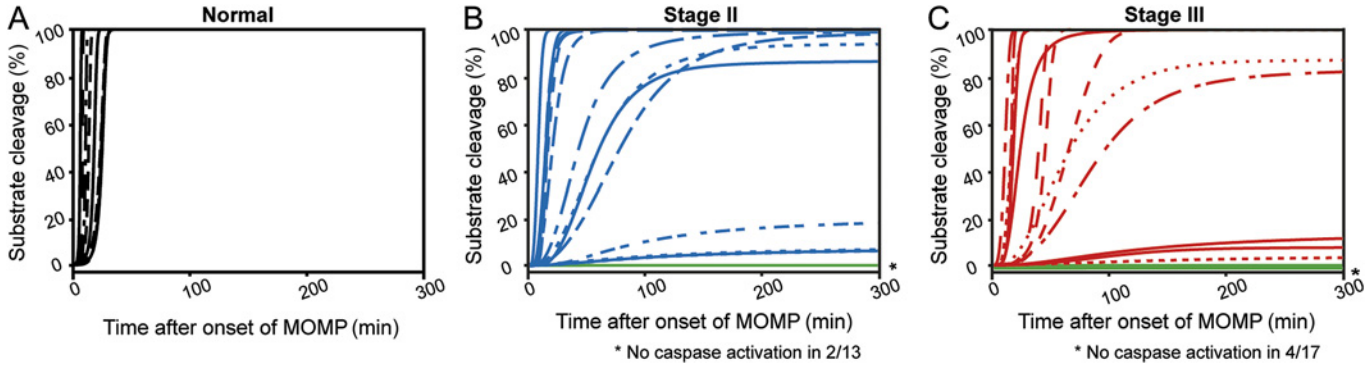

Figure 3 Systems model predicts decreased apoptosis execution with advancing disease stage. Model output in normal colorectal tissue (n=9) (A), (B) Stage II (n=13) (B), and Stage III (n=17) (C) colorectal tumours following implementation of protein concentrations. Graphs represent model output of caspase activation (per cent substrate cleavage) over time. Green lines indicate patients with no caspase activation predicted.

data extracted from primary colorectal tumours to predict the efficacy of 5FU-based chemotherapy on dormant micrometastatic sites. Twenty Stage II (n=3) and Stage III (n=17) patients in this study received adjuvant 5-FU- based chemotherapy (table 1). Of these patients, 12 were alive with no disease recurrence within 4 years follow-up (positive outcome) and eight had disease recurrence or disease mortality within 4 years (negative outcome).

Figure 5 shows the predicted ability of colorectal tumours to undergo effector caspase activation in response to MOMP for these patients. Modelling revealed that in 4 of the 20 patients, MOMP failed to induce any caspase activity (green traces in figure 5). Importantly, all four patients were in the group with negative clinical outcome. We also examined whether other output model parameters such as the maximal rate of cleavage and the percent substrate cleavage at which this slope was reached varied between patients with different clinical outcomes. Comparisons of maximum slopes of substrate cleavage between patients with positive and negative outcome indicated that negative outcome was associated with significantly lower caspase activity (Mann–Whitney U test; p=0.001). Similarly, there were significant differences in percent substrate cleavage at maximum slope (p=0.005) and percent substrate cleavage at 300 min (p=0.004).

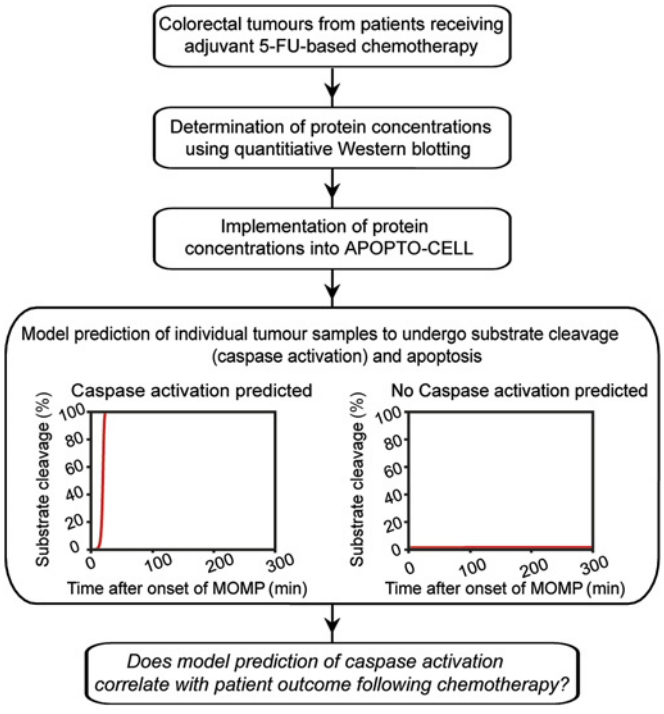

Figure 4 Schematic representation of study design. The APOPTO-CELL systems model was utilised to predict clinical outcome in colorectal cancer patients receiving 5-FU-based chemotherapy.

Previous studies from our laboratory indicated that cells with less than 25% caspase substrate cleavage following MOMP subsequently show no morphological changes characteristic of apoptosis.[3] Therefore, a threshold of 25% caspase substrate cleavage within 300 min of cytochrome-c release was used to examine the ability of the model to predict chemotherapy responsiveness. Using this criterion, apoptosis was predicted in 11 of 12 patients with positive outcome (figure 5A), while insufficient caspase activation was predicted in 6 of 8 patients with negative outcome (figure 5B); a predictive rate of 85% (17/20; Fischer exact test, p=0.005) and a specificity of 91.7%. In order to address whether the prediction rate of the model is affected by confounding variables such as the type of chemotherapy received or MSI status we performed a multivariate logistic regression. Of those patients who received chemotherapy, none of those assessed displayed MSI. Covariates in the analysis included model prediction of caspase activation, MSI status, tumour stage, tumour differentiation, age and gender. Of these co-variates, only model output was a significant predictor of patient outcome (OR=0.03, p=0.008).

To evaluate whether the above systems modelling results outperform statistical approaches commonly used in prognostic marker studies, we first examined the relationship between individual protein concentrations and response to therapy. Univariate statistical analysis indicated significantly higher XIAP expression in patients with negative outcome (Mann–Whitney U test; p=0.031) and significantly higher SMAC expression in patients with positive outcome (p=0.025), however discriminant analysis of these proteins alone indicate that neither individual proteins could sufficiently predict patient outcome on its own (p=0.170 and 0.197 respectively). None of the other proteins showed significant differences between patients with positive or negative outcome. In addition, we assessed whether expression of Pro-caspase 8, a key component of the extrinsic apoptotic pathway was associated with patient outcome. Western blotting indicated that there was no significant difference in Caspase 8 levels between normal and tumour tissue (Supplemental figure 5). Univariate statistics indicated that there was no difference between patients with positive and

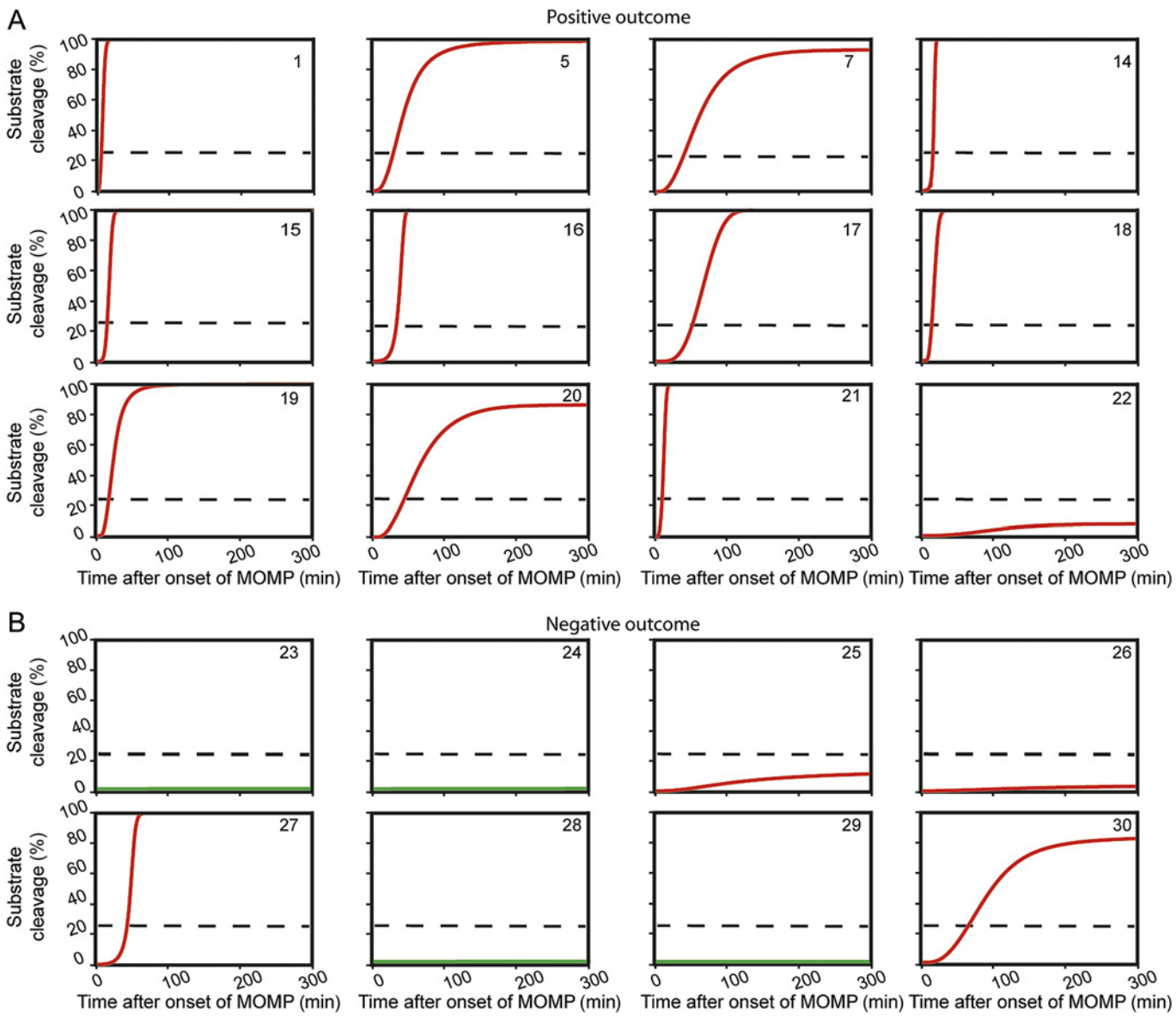

Figure 5 Model output correlates with treatment responses in colorectal cancer patients. Substrate cleavage profiles in individual colorectal cancer patients receiving chemotherapy with positive (n=12) (A) and negative outcome (n=8) (B). Graphs represent model output of caspase activation (per cent substrate cleavage) over time. Green lines indicate patients with no caspase activation predicted; dashed line indicates threshold of 25% substrate cleavage.

negative outcome (p=0.234) and discriminant analysis indicated that Procaspase 8 expression alone was not able to predict patient outcome following chemotherapy (p=0.298).

We next evaluated the ability of two multivariate statistical approaches, discriminant analysis and PCA, to determine the ability of proteins in combination to discriminate between patient outcomes. Discriminant analysis was employed to determine whether protein concentrations of APAF-1, XIAP, SMAC and Pro-Caspases 3 and 9 taken together were able to predict patient outcome. This method demonstrated while 75% (15/20) of patients were correctly classified, this method of analysis did not reach statistical significance (p=0.109). We also employed PCA to investigate whether a set of linear combinations of protein concentrations could distinguish between negative and positive outcomes. While 70% of the variance in the patient data set could be explained by the two major principal components (Supplementary figure 6A), no clustering of patient data with similar clinical outcome was observed (Supplementary figure 6B). Taken together these results indicate that the multivariate statistical approaches are outperformed in differentiating between patient outcomes by a systems approach which takes into account both signalling network topology and non-linear signalling dynamics.

Systems analysis approach as a predictive tool in determining individual responses to novel adjuvant therapies targeting apoptosis

The ability of this model to identify patients as unlikely to respond to 5-FU-based chemotherapy indicated that lack of chemotherapy responsiveness may be attributed to the inability of dormant metastatic tumour cells to efficiently execute apoptosis following MOMP. Therefore drugs which target the apoptosis pathway downstream of cytochrome-c release may have potential clinical utility in patients predicted as unlikely to

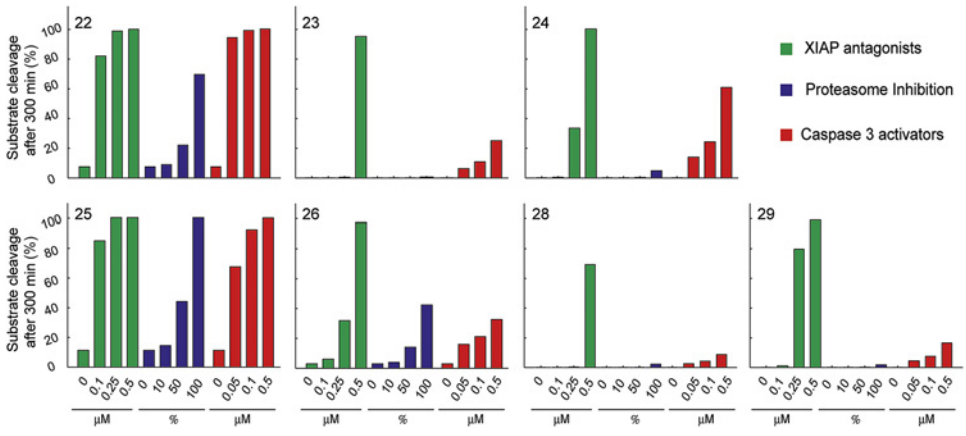

Figure 6 Predicting the effects of alternative, targeted treatment strategies in colorectal cancer patients. The APOPTO-CELL model was expanded to include the effects of XIAP antagonists (green bars), proteasome inhibition (blue bars) and direct activation of Pro-Caspase 3 (red bars). The effects of these drugs on caspase activation profiles in patients with <25% substrate cleavage predicted. Panels represent individual patients.

respond to traditional chemotherapies. XIAP antagonists might overcome chemoresistance by freeing processed, active caspases from their inhibitor XIAP.[20] Proteasome inhibition results in decreased protein degradation, a process important for proteins subject to enforced proteasomal degradation, in particular active Caspase 3 and 9. Direct activators of Pro-Caspase 3 result in activation of Caspase 3 independent of upstream Caspase 9,[22] thereby circumventing chemotherapy resistance due to decreased APAF-1 levels or high levels of XIAP.

We expanded the systems model to predict whether these drugs could re-establish Caspase 3 activity in those patients initially identified as unlikely to undergo apoptosis following chemotherapy (<25% substrate cleavage after 300 min). Interestingly, the model predictions indicated a marked variability in response to these agents between individual patients (figure 6). Proteasome inhibition and Pro-Caspase 3-activating compounds were predicted to be effective only in a subgroup of patients. In contrast, XIAP antagonists were found to be effective in all patients, yet at greatly varying concentration ranges, indicating the potential of this approach to provide guidance for personalised treatments for colorectal cancer patients.

DISCUSSION

Despite numerous studies aimed at identifying prognostic markers for colorectal cancer, tumour staging remains the key prognostic indicator, with other possible indicators including microsatellite instability[26] and tumour location.[27] In patients with non-metastatic colorectal cancer, adjuvant chemotherapy plays an important role in treatment: however the benefits even among this group are not entirely clear. Five year survival of Stage II patients is approximately 75% with surgery alone, therefore at maximum only 25% of these patients will potentially benefit from adjuvant chemotherapy. In Stage III patients, survival rates are low even with the introduction of 5-FU in combination with either oxaliplatin[1] or the topoisomerase I inhibitor irinotecan.[28] Chemoresistance limits effectively treating this disease, especially in more advanced disease stages. Identifying markers for therapy response is an integral component in decreasing mortality, as it will lead to personalised or less toxic therapies by differentiating between patients likely to respond to current chemotherapies and those more likely to respond to new targeted therapies. Considering the implications of dysregulated apoptosis in cancer, apoptotic regulatory proteins are frequently examined as potential biomarkers for patient prognosis and response to chemotherapy.[29] The majority of these studies focused on individual proteins or selections of proteins, yet attempts to incorporate system approaches taking into account signalling network topology as well as non-linear signalling dynamics have not yet been performed. This study highlights the advantages of systems biology approaches not only as tools for predicting chemotherapy response, but more importantly for determining alternative treatment strategies for patients unlikely to respond to traditional chemotherapeutics.

From the onset of our study, ODE-based modelling of effector caspase activation demonstrated that matched normal colon tissue from colorectal cancer patients would undergo rapid and complete substrate cleavage following MOMP. In comparison, tumours were predicted to have a diminished capacity to undergo substrate cleavage, in agreement with experimental evidence demonstrating decreased apoptosis susceptibility in tumour tissue compared with normal tissue.[30] The molecular mechanisms leading to altered protein levels of key apoptosis proteins in colorectal cancer include not only genetic but also epigenetic modifications.[31] For example, *apaf1* is hypermethylated in certain cancers[32] and is a target of the histone deacetylase HDAC2,[33] both of which lead to decreased APAF1 expression. Interestingly, comparisons of the mRNA levels and protein expression of the proteins examined showed little correlation. The lack of correlation between mRNA and protein levels highlights the importance of analysing protein levels in systems approaches which model pathways based on protein concentrations and their interactions. Similarly, other clinical studies have shown discordance between gene expression and protein production in prostate and lung cancers[34 35] with the question remaining as to why these differences exist. The mechanisms underlying translational regulation of these proteins may be important, including the initiation of cap-independent translation, which may allow for the translation of proteins which would normally be turned off in response to stress conditions.[36] In addition, miRNAs may negatively regulate translation, thereby leading to the decreased translation of protein from the mRNA template, independent of mRNA degradation.[37] Importantly, post-translational mechanisms such as different rates of protein degradation by the proteasome and cathepsins may also contribute to the lack of concordance between protein and mRNA expression.

As previously noted, the proteins examined here have previously been explored as prognostic markers in colorectal cancer. Studies have shown higher expression of caspase 3, SMAC and XIAP in colorectal tumours compared to matched normal tissue,[12 15] while Caspase 9 was present in lower levels in tumours,[16] with only XIAP, SMAC and APAF-1 having potential prognostic value using immunohistochemistry. Xiang *et al* demonstrated that expression of XIAP in tumours is associated

with poor prognosis.[11] Decreased expression of SMAC was associated with poor prognosis,[14] while increased expression of APAF-1 has been associated with better prognosis.[13 38 39] Using quantitative Western blotting techniques, we confirmed these previous studies, with patients with negative outcome following 5-FU-based chemotherapy having higher XIAP and lower SMAC concentrations. Yet as single markers these proteins were unable to discriminate between patients with negative and positive outcomes. Using the computational model of caspase activation however, we were able to predict response in patients treated with 5-FU-based chemotherapy in 85% (p=0.004) of patients, identifying those not responding to chemotherapy with 91.7% specificity. Apoptosis defects upstream of MOMP such as *bax* gene deletions[40] were not taken into account, however, they may also contribute to apoptosis resistance. Despite this limitation, the APOPTO-CELL approach was superior to the discriminant statistical analysis for classifying patients. Our study therefore demonstrates the suitability of a systems approach for predicting treatment responses, and its advantages over more traditional statistical approaches.

Importantly, our study demonstrates the feasibility of analysing the ability of primary tumour cells to undergo caspase activation in predicting responses of dormant micrometastatic cells to chemotherapy. By using primary tumour tissue, this study is limited in that it fails to take into account additional cellular changes occurring during metastasis. Given the difficulties of procuring sufficient quality tissue from metastatic sites, the high accuracy at which the APOPTO-CELL is able to predict outcome from the primary site is therefore an important finding. Furthermore, among those patients with negative outcome examined here, only 3 of 8 went on to have second line chemotherapy, all of whom went on to die from disease within the 4 year follow-up period. Therefore no real conclusions can be drawn as to the ability of the model to predict responses to second line therapy following recurrence. Future studies aimed specifically at validating the clinical utility of the APOPTO-CELL model in predicting combination therapy responses in metastatic CRC would shed light on the ability of the model to predict response to not only traditional chemotherapies, but also to targeted therapies currently in clinical practice such as the bevacizumab or cetuximab.

While 5-FU based adjuvant chemotherapies are currently the standard of care for colorectal cancer, survival rates still remain low and there is a pressing need to identify alternative treatments strategies for these patients. The present study highlights the importance systems biology based models may have in determining alternative treatment options which may prove to be effective strategies either alone or in combination with standard chemotherapies in patients who respond poorly to 5-FU based standard therapies. XIAP, SMAC, APAF-1, and Pro-Caspases 3 and 9 as well as the proteasome have been explored widely as viable therapeutic targets,[41–43] and drugs targeting these proteins are in clinical and pre-clinical testing.[44–46] The model indicated that the ability of these different drug classes to re-activate apoptosis may vary across patients, both in terms of drug type as well as effectiveness of each drug at fixed concentrations. Early stage clinical trials testing these novel agents may therefore avail of the approach taken in this study, and target individual patients for the inclusion into a trial. Limiting this however will be the paucity of suitable surgical resection samples with regard to both quantity and quality for protein analysis for immunoblotting as well as the time consuming nature of quantitative western blotting techniques. Therefore refinement and validation of this systems approach to potentially accommodate routine immunohistopathology data may represent an important future development. The simplicity of this approach, determining the protein levels of five key apoptosis proteins in resected tumour material relative to a HeLa cell standard, in combination with the APOPTO-CELL online tool[17] allows for easy incorporation into phase 2 clinical trials. While this approach has only so far been studied in colon cancer, it may be applied to a variety of cancers.

In conclusion, we here present the first proof-of-concept translational study examining the application of non-linear systems models in a clinical setting. We demonstrate that the APOPTO-CELL model has significant potential for use as a tool in identifying colorectal cancer patients unlikely to benefit from 5-FU based chemotherapy. In the future, such approaches may enable oncologists to make more informed treatment decisions, and to provide reasoning for the inclusion of alternative, adjuvant treatment paradigms.

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Clinical application of a systems model of apoptosis execution for the prediction of colorectal cancer therapy responses and personalisation of therapy

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